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(1) Hoffman et al. 1992, Anal. Biochem. Vol. 203, pp. 70-75

(2) Kienhaus et al. 1992, J. Receptor Res. Vol. 12, pp. 389-399

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SCINTILLATION PROXIMITY ASSAY TO STUDY THE INTERACTION OF EPIDERMAL GROWTH FACTOR WITH ITS RECEPTOR

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ABSTRACT

Scintillation Proximity Assay (SPA), which does not require the physical separation of receptor bound and free ligand, was applied to study the interaction of Epidermal Growth Factor (EGF) with its receptor (EGFR) in membrane preparations from human placenta. Fluomicrospheres to which the monoclonal anti-EGFR antibody R1 was coupled, were used. Kinetic binding data of the association of ^{125}I-labeled EGF binding to the receptor at 20 °C could be fitted according to a double exponential model, which is consistent with the presence of fast and slow associating EGF binding sites. Dissociation kinetics revealed that perturbation of equilibrium conditions rapidly occurs upon washing. Multiple point Scatchard analysis of equilibrium ^{125}I-labeled EGF binding data revealed curvilinearity, indicating the presence of both high and low affinity EGF binding sites. We conclude that SPA is an interesting new tool in the exploration of the interaction of ligands with their receptors, which allows detailed ligand-receptor studies under precise in situ conditions.

INTRODUCTION

Common procedures for investigation of ligand-receptor interactions involve equilibration of radiolabeled ligand with receptor-containing preparations followed by the physical separation of receptor-bound and free ligand. The unavoidable drawback of these separation methods is the disturbance of equilibrium conditions, which could result in misinterpretation of the binding data.

Scintillation Proximity Assay (SPA), introduced by Hart and Greenwald in 1979 (1), is based on the activation of a fluorophor integrated in microspheres by ^{3}H- or ^{125}I-labeled ligands which are in close proximity to the beads by binding to covalently or hydrophobically coupled acceptor molecules (1-4). Because free ligand itself does not induce photon emission, separation of bound and free radioligand becomes superfluous (4). In spite of this advantage, only a limited number of studies concerning SPA, which are mainly dealing with application in radioimmunoassay, emerged in the literature (3,5). In one study, SPA has been applied for monitoring the interaction of the acetylcholine receptor with ^{125}I-labeled α-bungarotoxin (6).

In the present report SPA was employed to study the interaction of Epidermal Growth Factor (EGF) with its receptor (EGFR), present in membranes of human placenta. In this application, the SPA methodology is extremely suitable in studies on ligand-receptor binding kinetics, since the amount of binding can be monitored following mixing of the SPA beads with the receptor preparation and the radioligand used, without perturbation of equilibrium conditions.

MATERIALS AND METHODS

Mouse monoclonal EGFR-antibody R1 (7) coupled polyvinyltoluene (PVT) Scintillation Proximity Assay (SPA) beads were obtained from

Amersham International plc., Cardiff, U.K. Receptor grade mouse EGF was obtained from Bioproducts for Science, Indianapolis, IN, USA. Carrier free $Na^{125}I$ was purchased from Amersham International plc., Buckinghamshire, U.K.

Methods

EGF was radioiodinated using Enzymobeads (Bio-Rad Laboratories, Richmond, CA, USA) to a specific radioactivity of about 500 Ci/mmol (8). Membranes from human placenta were obtained by a previously described procedure (8).

One volume of Human Placental Membranes (HPM), which contained approximately 10,000 fmoles of EGFR protein/ml, was incubated with 10 volumes of beads suspension containing 10 mg of SPA beads per ml of buffer (20 mM phosphate, 150 mM NaCl, 50 μM bacitracin, pH 7.4), in 3 ml polyethylene vials for 16 h at 20 °C under continuous agitation. 100 μl of this beads-membranes suspension was brought into a miniscintillation vial (Milli-6 vials, Lumac BV, The Netherlands) containing ^{125}I-labeled EGF to start the binding reaction in final volume of 240 μl. The total amount of ^{125}I-labeled EGF added was measured in a LKB-Wallac (Turku, Finland) 1261 multi gamma counter. After overnight equilibration at 20 °C, bound ^{125}I-labeled EGF was assessed by scintillation counting in a Canberra Packard (Downers Grove, IL, USA) Tri-Carb 1600 TR Liquid Scintillation Counter. For kinetic studies, the vials were placed in the scintillation counter immediately following the initiation of the reaction and were monitored by scintillation counting continuously every minute for 99 minutes.

Scatchard analysis (9) was performed by nonlinear, weighted regression analysis, employing Marquardt's algorithm (10) for fitting of the binding data. Radioiodinated fluomicrospheres were used as calibration standards to

enable conversion of the counts from the gamma and scintillation counter, The conversion factor was determined as the ratio of gamma counts/scintillation counts, and was 1.95.

RESULTS AND DISCUSSION

Since the discovery of EGF in 1962 (11), many studies were performed investigating the interaction of this growth factor with its receptor. However, all these kinetic and equilibrium studies involved the separation of receptor-bound and free EGF. We used SPA to investigate the kinetics of EGF binding to its receptor in human placental membranes and to investigate equilibrium binding parameters under nondissociating conditions. The described SPA involved the use of fluomicrospheres coated covalently with monoclonal anti-EGFR antibodies, which enabled binding of EGFR prior to the addition of ^{125}I-labeled EGF to initiate the ligand receptor reaction.

Fig. 1 shows the effect of increasing amounts of fluomicrospheres in the assay. The signal from ^{125}I-labeled EGF binding to the membranes appeared to increase with increasing amounts (up to 1 mg) of fluomicrospheres. The maximum specific signal was observed when 1 mg of beads or more were used per assay tube. This indicates that all membranes were bound to the beads under these particular circumstances. In subsequent experiments we used 1.5 mg of beads per assay tube.

Association binding of two different ^{125}I-labeled EGF concentrations to the beads-coupled EGFR is shown in Fig. 2. Both for high and low ^{125}I-labeled EGF concentrations, there was a very fast increase in EGF binding in the first few minutes of incubation, followed by a gradually increase of the signal during further incubation. The complete set of recorded kinetic binding data in Fig. 2 could be fitted according to a double exponential model, which is consistent with the presence of fast and slow associating

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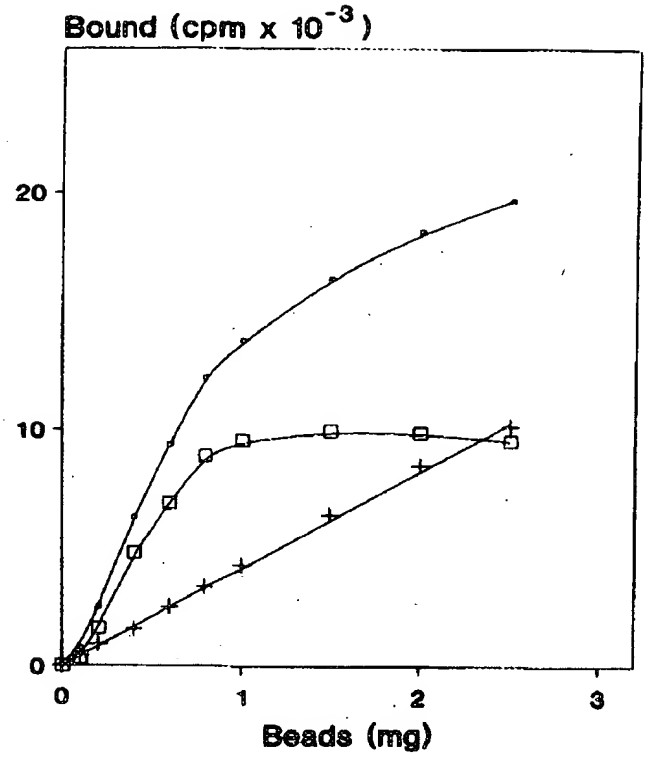

Fig. 1. Effect of increasing concentrations of fluomicrospheres on the amount of ^{125}I-labeled EGF binding. Counts bound were measured in the presence (▣) and in the absence of membranes (+). The signal from ^{125}I-labeled EGF binding to the membranes was calculated as the difference between the total and the background signal (□).

EGF binding sites in the HPM preparation. The exponential character of association kinetics in the first part of the figure is due to the few data points very difficult to determine. However, the association rate constant of the second exponential was about 7 x 10^6 $M^{-1}.min^{-1}$ at 3.5 nM EGF, which is of the same order of magnitude as other reported values (12).

Since the slow increase in binding in these experiments may be due to a slow increase of the nonspecific binding during incubation, the association kinetics of the nonspecific ^{125}I-labeled EGF binding were investigated in the

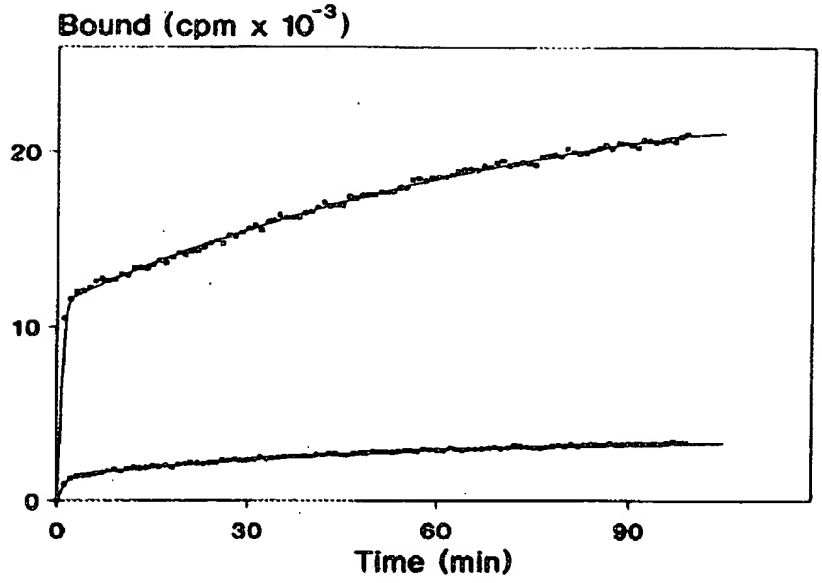

Fig. 2. Association kinetics of binding of 0.25 nM (lower dots) and 3.5 nM (upper dots) ^{125}I-labeled EGF to beads coupled membranes from human placenta.

presence of 1 μM of unlabeled EGF and subsequently subtracted from the original association binding data. The resulting specific association kinetics could also be fitted to a double exponential model. These two EGF-binding moieties with different association kinetics were also observed in association binding studies, in which the pre-equilibrated beads and membranes were washed twice with one volume of assay buffer prior to the addition of EGF to remove unbound membranes (data not shown). In the latter experiments the bound signal was lower as compared to the nonwashed beads, which implies that some of the EGFR-containing membranes dissociate from the beads upon washing.

Dissociation kinetics of the EGF binding were studied after the addition of 900 μl of assay buffer with or without 1 μM EGF to 100 μl of beads-coupled ^{125}I-labeled EGF-receptor complex. Fig. 3 shows that due to dilution

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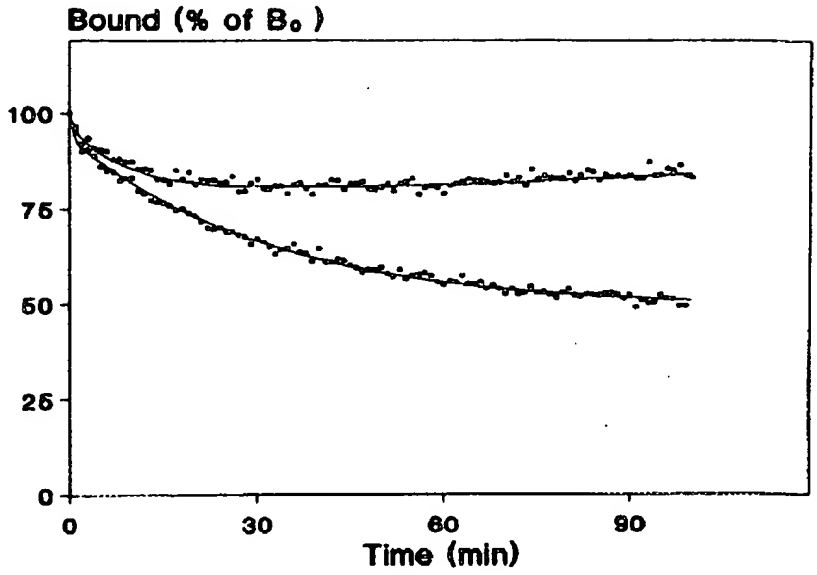

Fig. 3. Dissociation kinetics of ^{125}I-labeled EGF from HPM, which was initiated by the addition of 9 volumes of assay buffer in the absence (upper dots) or presence of excess (1 μM) unlabeled EGF (lower dots). The initial dissociation rate constant was $\approx$0.03 min^{-1}.

with buffer only, a new equilibrium situation was rapidly reached within 30 min. Moreover, in Fig. 3 is shown that approximately 50% of the initially bound ^{125}I-labeled EGF dissociates in about 60 min after addition of excess of unlabeled EGF. Plotting $\ln(B_t/B_0)$ versus time resulted in a curvilinear graph, even in the presence of 1 μM of unlabelled EGF, which is consistent with the presence of two different EGF receptor classes. The initial dissociation rate constant was found to be $\approx$0.03 min^{-1}. Dilution with assay buffer only did not result in a release of the EGFR-containing membranes from the antibody on the fluomicrospheres, to which they were initially bound. This was established in parallel experiments by centrifuging the diluted fluomicrospheres, and assay of the supernatant fraction for the presence of EGFR by radioligand binding assay according to the previously

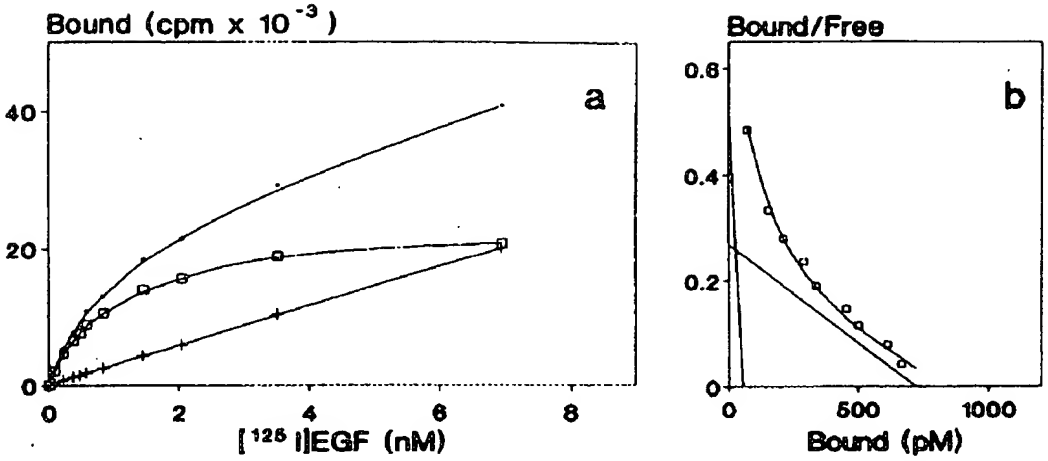

Fig. 4. a. Effect of increasing concentrations of ^{125}I-labeled EGF binding to HPM (■ : total binding, +: nonspecific binding, and □: specific binding) by SPA. Nonspecific binding was measured in the presence of 250-fold excess of unlabeled EGF.
b. Scatchard plot of specifically bound ^{125}I-labeled EGF to EGFR-containing HPM.

described hydroxylapatite procedure (13). The rapid establishment of a new equilibrium by dilution with assay buffer suggests that misleading binding data will be obtained in commonly used EGFR ligand binding assays, since in these methods extensive dilution/washing steps are involved in the separation of receptor bound and free radioligand.

Fig. 4 displays saturation analysis of the binding of ^{125}I-labeled EGF to HPM using SPA (Fig. 4a). Increasing concentrations of ^{125}I-labeled EGF in the absence (total binding) and in the presence of 1 μM unlabeled EGF (nonspecific binding) were added to the beads-membranes suspension. It is demonstrated that saturation of EGF binding to the beads occurs at EGF concentrations of approximately 5 nM at 20 °C, during incubation for 15-20 hours. The ^{125}I-labeled EGF binding in the presence of excess unlabeled EGF accounted for 5% or less of the total radioactivity added. The ^{125}I-labeled

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EGF binding in the absence of membranes was approximately 2% of the total radioactivity added. Transformation of the binding data revealed curvilinear Scatchard plots, indicating the presence of both high and low affinity EGF binding sites which were bound to the R1 antibody (Fig. 4b). The apparent dissociation constants (Kd) were about 40 and 600 pM for the high and the low affinity sites respectively.

The described SPA method includes a 11-fold dilution of the membranes in the coupling reaction. In the case of HPM, which contains large amounts of EGFR protein, this dilution does not result in an unmeasurable amount of receptors. However, membranes containing small amounts of EGFR protein, such as membranes from human tumors, do not permit to perform such an additional dilution step of the sample prior to the assessment of EGFR. We therefore conclude that, in these particular assay conditions, the described SPA multiple point ligand binding assay is less sensitive as compared to regular EGFR assays, in which separation of receptor-bound and free ligand is included. Moreover, the nonspecific binding and the background signal (blank) were higher in the SPA assay (5% and 2% respectively) than in regular EGFR ligand binding assays. The higher nonspecific binding and background signal observed in SPA might be the result of the activation of the fluorophor in the beads by some ligand molecules, which are in close proximity but are not bound to the bead (nonproximity effect).

However, SPA methodology confers many unique and significant advantages for measurements of kinetics of ligand-receptor kinetics. Since bound ligands produce light, but free ligands do not, the need for any of the traditional tedious separation procedures is obviated, so that the in situ situation is not disturbed. This system is therefore applicable for on-line monitoring of the kinetics of ligand receptor binding and to study binding under absolute equilibrium conditions. For quantitative assessment of EGF receptors by SPA, it could be possible to use an EGFR calibration curve, since the signal appears to be linearly related to the amount of EGFR

protein added (data not shown). In the case of acetylcholine receptors, as little as 1 ng could be detected by SPA (6).

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